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Expression of an uncoupling protein gene homolog in chickens

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Abstract

An avian uncoupling protein (UCP) gene homolog was recently sequenced from skeletal muscle and was proposed to have a role in thermogenesis in chickens, ducks and hummingbirds. Since mammalian UCP 2 and UCP 3 also appear to have functions associated with energy and substrate partitioning and body weight regulation, the purpose of this study was to further characterize chicken UCP under conditions of nutritional stress and/or leptin administration. Male 3-week-old chickens were starved for 24 or 48 h and then half of each group was refed for an additional 24 h. In a follow-up experiment, chickens were fed or starved for 48 h with or without leptin administration. Feed deprivation increased UCP mRNA expression in skeletal muscle by up to 260% ($P<0.001$), and in a time-dependent manner in pectoralis muscle. Refeeding for 24 h normalized muscle UCP mRNA levels. Leptin administration had no effect on muscle UCP. Chicken muscle UCP mRNA levels were highly correlated with plasma triglyceride and non-esterified fatty acid (NEFA) concentrations, and with circulating levels of insulin, insulin-like growth factor (IGF)-I and IGF-II. These results suggest that, as in mammals, avian UCP is up-regulated during feed deprivation and is highly correlated with increased fatty acid oxidation and flux into skeletal muscle.

Keywords: Chicken; Feed deprivation; Free fatty acids; Insulin-like growth factor (IGF); Leptin; Refeeding; Skeletal muscle; Triglyceride; Uncoupling protein

1. Introduction

Uncoupling proteins are members of the mitochondrial membrane transporter family. UCP 1 is expressed in brown adipose tissue in rodents and plays an important role in adaptive thermogenesis by dissipating the mitochondrial transmembrane proton gradient and producing heat (Himms-Hagen, 1985). Four additional uncoupling protein homologs have been identified to date. UCP 2 is ubiquitously expressed (Fleury et al., 1997), while UCP 3 gene expression is mainly found in skeletal muscle, adipose tissue and heart (Acin et al., 1999). UCP 4 (Mao et al., 1999) and brain mitochondrial carrier protein type 1 (BMCP 1, Sanchis et al., 1998) have been more recently identified and both are mainly expressed in the brain and neural tissues.

Most of the functional characterization of these UCPs to date has been conducted in mammals. Recently, a UCP homolog was identified in chickens, ducks (Raimbault et al., 2001) and hummingbirds (Vianna et al., 2001), and appears to play a role in adaptive thermogenesis. Gene expression of avian UCP in skeletal muscle was induced by cold exposure and glucagon treatment in ducks (Raimbault et al., 2001), while UCP expression in hummingbird cardiac muscle was increased during

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torpor (Vianna et al., 2001), a condition similar to an overnight hibernation. These three conditions are all associated with non-shivering thermogenesis in birds. Golozoubova et al. (2001) have evidence from ablated mice that only UCP 1 can mediate non-shivering thermogenesis during cold acclimation. However, the nucleic acid sequence of avian UCP is highly homologous to both mammalian UCP 2 and UCP 3. The current hypothesis on the function of these two UCPs involves regulation of fatty acid oxidation, especially during times of metabolic stress (Dulloo and Samec, 2001), possibly by transporting fatty acid ions (Himms-Hagen and Harper, 2001) out of the mitochondria. Interestingly, cold acclimation and glucagon administration to ducklings both resulted in increased plasma NEFA and glycerol levels (Benistant et al., 1998). Fatty acid oxidation is known to be the primary energy source for fasted, resting hummingbirds (Suarez et al., 1990).

In mammals, UCP 2 and UCP 3 mRNA levels are modulated by fasting (Samec et al., 1998; Spurlock et al., 2001) and refeeding (Marzolo et al., 2000), and leptin (Combatsiaris and Charron, 1999). Feed deprivation increases skeletal muscle gene expression of UCP 2 and UCP 3 through the elevation of serum free fatty acid (FFA) concentrations (Samec et al., 1998). Chronic exogenous leptin exposure reduces adipose tissue mass and increases energy expenditure (Halaas et al., 1997), which is partially mediated by UCP 1. In mouse skeletal muscle, short-term leptin infusion decreases UCP 3 (Combatsiaris and Charron, 1999), whereas in rats, chronic leptin exposure increases UCP 3 gene expression (Cusin et al., 1998). Again, this up-regulation of muscle UCP 3 by chronic leptin exposure could also be influenced by elevated FFA levels.

The interaction between insulin, IGF-I and UCPs has previously been shown in streptozotocin-induced diabetic rats. Kageyama et al. (1998) reported elevated gene expression of both UCP 2 and UCP 3 in gastrocnemius muscle of diabetic rats, which was restored to normal after insulin treatment. However, diabetes in rats (Houwing et al., 1995) is also associated with enhanced fat oxidation. Humans with type II diabetes exhibit reduced UCP 3 expression in skeletal muscle (Krook et al., 1998) and mutations in the UCP 3 gene have been discovered in some forms of non-insulin-dependent diabetes (Argyropoulos et al., 1998). In addition, diabetic rats exhibited decreased mRNA transcripts for IGF-I and its receptor (Bitar et al., 1997). Recently, Gustafsson et al. (2001) reported that UCP 3 can be up-regulated by both IGF-I and its receptor.

The present study was undertaken to determine the effects of fasting and refeeding, and fasting in combination with sustained leptin administration on avian skeletal muscle UCP, along with any possible interactions/correlations between UCP expression and plasma hormones and metabolites, most notably free fatty acids.

2. Materials and methods

2.1. Animals

Male 1-day-old broiler chicks were purchased from Shaver Poultry Breeding Farms (Cambridge, Ontario, Canada). The birds were group-housed in batteries in an air-conditioned room. They were maintained on a commercial ration (18% crude protein) and water was provided ad libitum before the experiments. All protocols involving the use of animals were approved by the Beltsville Animal Care and Use Committee.

2.2. Experiment 1

Eight chickens, at 8 weeks of age, were decapitated and samples of pectoralis major muscle, liver, heart, kidney, adipose tissue, spleen, brain and lung were dissected. Tissue samples were wrapped in aluminum foil, flash-frozen in liquid nitrogen and stored at −80 °C until RNA could be extracted.

2.3. Experiment 2

A total of 30 chickens were randomly allotted to five treatment groups at 3 weeks of age. Treatments groups were: control, ad libitum fed; feed-deprived for 24 h; feed-deprived for 24 h and then refed for 24 h; feed-deprived for 48 h; or feed-deprived for 48 h and then refed for 24 h. All six birds in a treatment group were penned together with unlimited access to water. At the termination of the treatment periods, birds were decapitated and samples of pectoralis major muscle and iliotibialis muscle were excised. Samples were wrapped in aluminum foil, flash-frozen in liquid nitrogen and stored at −80 °C until RNA could be extracted.

2.4. Experiment 3

A total of 20 birds were allotted to four treatment groups at 3 weeks of age. Treatment groups were: control, ad libitum fed and injected intraperitoneally (i.p.) with 100 μl of sterile-filtered phosphate-buffered saline (PBS); leptin, ad libitum fed and injected i.p. with 100 μg of recombinant human (rh) leptin (Calbiochem, La Jolla, CA, USA); feed-deprived for 48 h and i.p. injected with PBS; or feed-deprived for 48 h and i.p. injected with leptin. All birds were injected a total of five times; twice daily at 09:00 h and 15:00 h for 2 consecutive days, and again at 09:00 h on day 3. All five birds in a treatment group were penned together with unlimited access to water. Feed was removed from the two feed-deprived groups just prior to the first injection. Feed was weighed for the two fed groups on a daily basis. Starting at 10:00 h on day 3, the birds were bled via cardiac puncture and then decapitated. Pectoralis major and iliotibialis muscle samples were dissected out, wrapped in aluminum foil, flash-frozen in liquid nitrogen and stored at −80 °C until RNA could be extracted. All chickens were killed by 12:00 h. Cumulative feed consumption was also determined.

2.5. Plasma chemistry and RIAs

Blood samples were collected in EDTA-treated tubes, allowed to clot, and spun at 2800×*g* for 30 min. Plasma glucose and lactate were determined using commercial chemistry kits (YSI Biochemistry Analyzer, 2700 Select). Non-esterified fatty acid (NEFA) kits were purchased from Wako Chemical (Richmond, VA, USA) and triglyceride was determined using a Sigma kit (St. Louis, MO, USA). Double-antibody radioimmunoassays were used to determine plasma concentrations of the following hormones: insulin, intraassay coefficient of variation of 2.9% (McMurtry et al., 1983); insulin-like growth factor-I (IGF-I), intraassay coefficient of variation of 2.6% (McMurtry et al., 1994); chicken IGF-II, intraassay coefficient of variation of 3.6% (McMurtry et al., 1998), chicken leptin, intraassay coefficient of variation of 2.2% (McMurtry, unpublished). The cross-reactivity of rh leptin and mouse leptin in the chicken leptin assay is 0.25 and 2.0%, respectively. Plasma glucagon and rh leptin were determined utilizing kits purchased from Linco Research Inc, (St. Charles, MO, USA). Plasma used for the glucagon determination contained 1000 IU Trasylol.

2.6. RT-PCR assay of UCP

Total RNA was isolated from frozen tissue samples using the Trizol procedure (Life Technologies, Gaithersburg, MD, USA). Reverse transcription (RT) was carried out using the SuperScript II first-strand synthesis system for RT-PCR (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions, with 50 ng of random hexamers and 1 μg of total RNA. Polymerase chain reaction (PCR) amplification of cDNA was performed with Life Technologies (Gaithersburg, MD, USA) components in a 25-μl reaction volume containing: 2.5 μl of 10× PCR buffer; 1 μl of 50 mM $MgCl_2$; 0.5 μl of 10 mM dNTP mix; 1 unit of Platinum *Taq* DNA polymerase; 1 μl of cDNA; 1 μl of a 10 μM mix of sense (5′-GTGGATGCCTACAGGACCAT-3′) and antisense (5′-ATGAACATCACCACGTTCCA-3′) primers for chicken UCP (GenBank Accession No AF287144); and, depending on the application, duplexed with or without 1 μl of a 10 μM mix of sense (5′-TGCGTGACATCAAGGAGAAG-3′) and antisense (5′-TGCCAGGGTACATTGTGGTA-3′) primers for chicken β-actin (GenBank Accession No L08165). PCR was performed in a Peltier thermal cycler (MJ Research, Waltham, MA, USA, PTC-200) under the following conditions: one cycle at 94 °C for 2 min; 30 cycles at 94 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min; and a final extension cycle at 72 °C for 8 min. This gave a chicken UCP product of 389 bp and a β-actin product of 300 bp. All chicken UCP RT-PCR samples were in the linear range of logarithmic amplification without primer limitation. Dilution analyses of samples with high and low mRNA levels resulted in an average of 90% of predicted ratios. To eliminate the possibility of genomic DNA contamination, stringent DNAse I treatment (DNA-*free* kit, Ambion, Austin, TX, USA) was performed on the RNA from the eight different chicken tissues examined. The chicken UCP 389 amplicon (389 bp) was excised from an agarose gel for each of the eight tissues, re-amplified, and run through a GenElute PCR clean-up kit (Sigma Chemical Co, St. Louis, MO, USA). Sequences were confirmed by automated fluorescent DNA sequencing (ABI 310, Perkin Elmer Applied Biosystems, Foster City, CA, USA and/

or CEQ 2000XL, Beckman Coulter, Fullerton, CA, USA). All RT-PCR products were quantified by capillary electrophoresis with laser-induced fluorescence (CE-LIF). Samples were diluted 1:100 in deionized water prior to analysis by CE-LIF as previously described (Richards et al., 2000) on a P/ACE MDQ (Beckman Coulter, Fullerton, CA, USA) equipped with an argon-ion LIF detector. The peak areas were calculated by P/ACE MDQ software (Beckman Coulter, Fullerton, CA, USA). The peak area ratios of UCP/β-actin were determined for each RT-PCR sample and used as a measure of relative gene expression.

2.7. RT-PCR assays of IGF-I and IGF-I receptor

RNA isolation and reverse transcription were performed as for UCP above. PCR amplification of cDNA was performed with Life Technologies (Gaithersburg, MD, USA) components in a 25-μl reaction volume containing: 2.5 μl of 10× PCR buffer; 1 μl of 50 mM $MgCl_2$; 0.5 μl of 10 mM dNTP mix; 1 unit of Platinum *Taq* DNA polymerase; 1 μl of cDNA; 1 μl of a 10 μM mix of either sense (5′-GCTGAGCTGGTTGATGCTCT-3′) and antisense (5′-CACGTACAGAGCGTGCAGAT-3′) primers for chicken IGF-I (GenBank Accession No M32791), or sense (5′-CAAGCATGCGTGAGAGGATA-3′) and antisense (5′-CAAACCTTTCCCTCCTTTCC-3′) primers for chicken IGF-I receptor (GenBank Accession No AJ223164), duplexed with either 0.2 μl (for IGF-I) or 0.133 μl (for IGF-I receptor) of a 10 μM mix of the same sense and antisense primers used for chicken β-actin in the UCP assay. PCR was performed in a Peltier thermal cycler (MJ Research, Waltham, MA, USA, PTC-200) under the following conditions: one cycle at 94 °C for 2 min; 35 cycles at 94 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min; and a final extension cycle at 72 °C for 8 min. This gave a chicken IGF-I product of 202 bp and an IGF-I receptor product of 404 bp. Sequences of chicken IGF-I, IGF-I receptor and β-actin amplicons were confirmed by automated fluorescent DNA sequencing (ABI 310, Perkin Elmer Applied Biosystems, Foster City, CA, USA and/or CEQ 2000XL, Beckman Coulter, Fullerton, CA, USA). All RT-PCR products were quantified by capillary electrophoresis with laser-induced fluorescence (CE-LIF) as described above for UCP.

2.8. Statistics

The data were analyzed by either one-way (Experiment 2) or two-way (Experiment 3) analysis of variance using the General Linear Models (GLM) procedure of SAS. Data are presented as the mean ± S.E.M. Means were further compared with Bonferroni's test for multiple comparisons. Spearman simple correlations were also determined for all parameters measured in Experiment 3. Significance was set at $P < 0.05$.

3. Results

3.1. Tissue distribution of chicken UCP

All eight tissues examined exhibited some degree of UCP expression (Fig. 1), as determined by RT-PCR. Chicken UCP was highly expressed in skeletal muscle. Spleen and brain tissue had moderate levels of UCP expression. Kidney, heart and lung exhibited slight UCP expression, while liver and adipose tissue appeared to have the lowest expression of UCP of all tissues examined. The identities of all eight PCR products were confirmed as chicken UCP by direct sequencing. The presence of additional PCR product bands for all tissues, excluding skeletal muscle, was observed. The consistent band above 1000 bp was excised, but would not re-amplify under our conditions, and thus its identity could not be determined by DNA sequencing.

3.2. Effects of feed deprivation and refeeding on chicken UCP

The CE-LIF analysis of the PCR samples (Fig. 2) indicated that chicken UCP gene expression is increased by feed deprivation in iliotibialis (thigh) muscle by up to 150%, and that this increase is time-dependent in pectoralis (breast) muscle (Fig. 3). Refeeding for 24 h returns UCP expression levels to normal in these muscles. Re-introduction of feed following a period of feed deprivation may actually reduce UCP mRNA levels below those of ad libitum fed controls, since although not significant, iliotibialis muscle UCP expression was 48% lower than that of ad libitum fed controls after 24 h of feed deprivation.

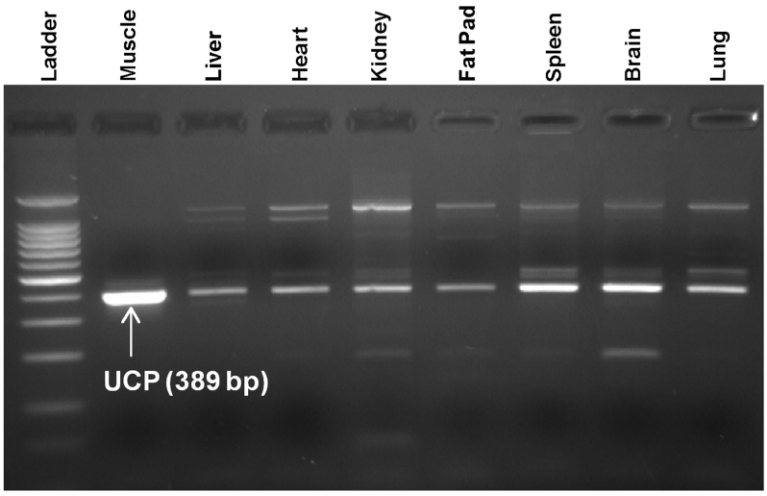

Fig. 1. Tissue distribution of avian UCP (389 bp) mRNA in chicken tissues. Expression was investigated by RT-PCR. Lane 1 is a 100-bp ladder, with the bright middle band representing 500 bp. Lanes 2–9 are pectoralis muscle, liver, heart, kidney, adipose tissue, spleen, brain and lung, respectively.

3.3. Effects of feed deprivation and/or leptin treatment on chicken UCP expression, plasma hormones and metabolites

Feed deprivation in chickens again caused an increase in skeletal muscle UCP expression of up to 130% in pectoralis muscle and 160% in iliotibialis muscle (Fig. 4). Daily leptin administration for 48 h had no effect on muscle UCP, either in fully fed birds or in those deprived of feed for 48 h.

Plasma rh leptin levels were undetectable in the uninjected birds, while the feed-deprived chickens had 37% higher concentrations of rh leptin than fed birds (Table 1). Endogenous chicken leptin was increased by up to 28% on rh leptin treatment. Administration of rh leptin had no effect on total feed consumed over the 2-day period (1160 g for the control pen vs. 1133 g for the leptin-treated pen). Leptin treatment alone in fed birds increased plasma triglyceride and IGF-I concentrations, without affecting NEFA or IGF-II levels. Lactate concentrations were elevated 60% by leptin administration, but due to high variability among birds in this treatment group, this increase was not significant ($P>0.055$) compared to controls. Plasma glucose levels were unaffected by rh leptin treatment. Glucagon concentrations were decreased 50% by rh leptin administration, but again due to variability in the fed control group of birds, this finding was not significant ($P>0.075$). Plasma insulin levels were increased by administration of rh leptin, by 42% in fasted and 70% in fed birds.

Feed deprivation led to decreases in plasma triglyceride concentrations of 50%, with a concomitant elevation in NEFA levels of 180% (Table 1). Plasma insulin concentrations were also decreased by 50% during fasting. Plasma IGF-I levels dropped by up to 50% after feed removal, while IGF-II concentrations increased by 40%. IGF-I gene expression in pectoralis muscle decreased by 33% after feed deprivation, proportional to the decline observed in plasma IGF-I levels, while muscle IGF-I receptor mRNA increased by 33% (Fig. 5). Feed deprivation had no effect on glucose, lactate or glucagon levels.

Chicken UCP mRNA levels between the two different skeletal muscles examined were highly correlated ($r=0.85$; Table 2). Both muscle UCP mRNAs were also positively correlated with circulating levels of NEFA ($r=0.70$), IGF-II ($r=0.58$) and muscle IGF-I receptor mRNAs ($r=0.65$), and negatively correlated with plasma triglyceride ($r=-0.79$), insulin ($r=-0.71$), IGF-I ($r=-0.80$) and muscle IGF-I expression ($r=-0.74$). Pectoralis muscle UCP mRNA was also correlated with plasma glucagon ($r=0.56$) and chicken leptin ($r=-0.46$) concentrations.

Rh leptin concentration was only correlated with lactate ($r=0.50$) and chicken leptin ($r=0.56$; Table 3) concentrations. Plasma triglyceride and NEFA levels were negatively correlated with each

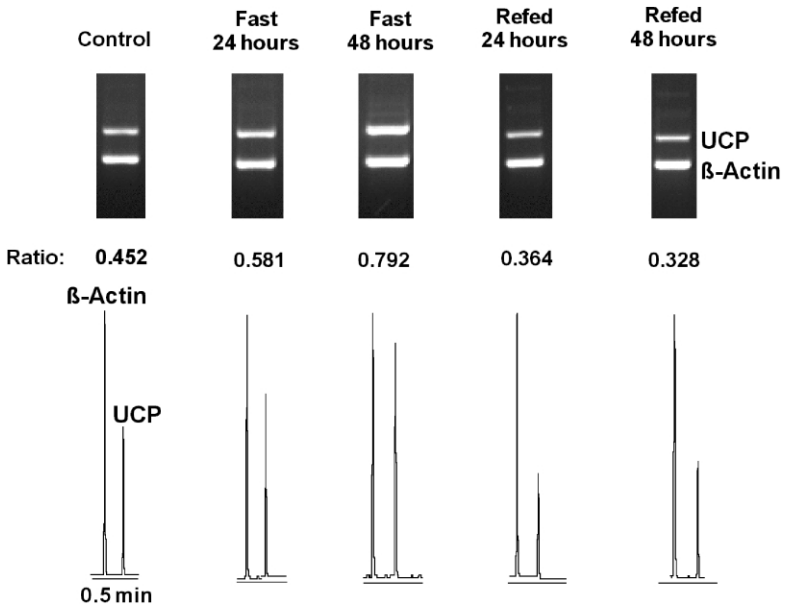

Fig. 2. The upper panels represent UCP mRNA expression (389-bp upper band) in pectoralis major muscle in control chickens, in those fasted for 24 or 48 h, or chickens refed for 24 h after a 24- or 48-h fast. Expression was investigated by RT-PCR with β-actin (300-bp lower band) as the internal standard. The ratio is the integrated peak area for UCP/β-actin. The bottom inserts show each of the above PCR products after analysis by capillary electrophoresis, with the left peak representing β-actin and the right peak representing UCP.

other ($r=-0.76$), and both were inversely correlated with insulin ($r=0.73$ for triglyceride; $r=-0.45$ for NEFA), IGF-I ($r=0.79$ for triglyceride; $r=-0.74$ for NEFA) and IGF-II concentrations ($r=-0.64$ for triglyceride and $r=0.56$ for NEFA), pectoralis muscle IGF-I ($r=0.83$ for triglyceride and $r=-0.68$ for NEFA) and pectoralis muscle IGF-I receptor gene expression ($r=-0.68$ for

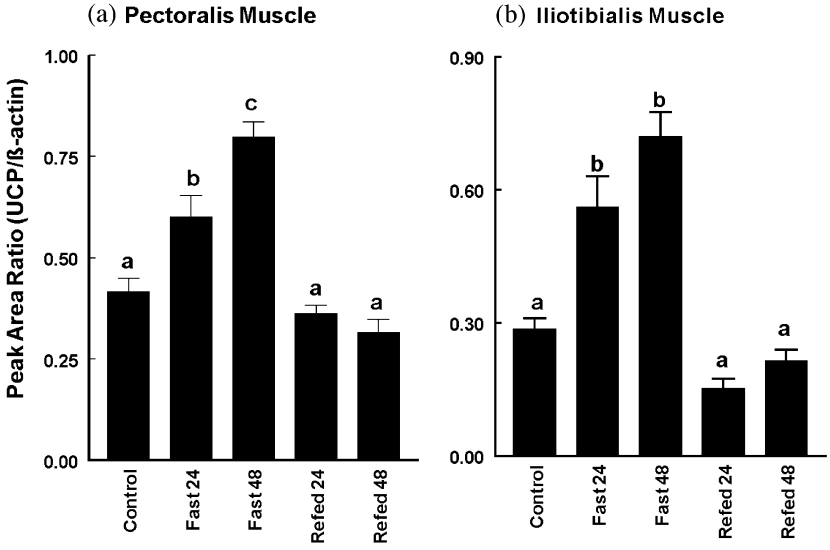

Fig. 3. Effect of feed deprivation for 24 or 48 h, or subsequent refeeding for 24 h, on changes in chicken UCP gene expression as measured by the integrated peak area ratio of UCP/β-actin in either (a) pectoralis major or (b) iliotibialis muscle. Values are plotted as mean ± standard error ($n=6$). Error bars with different letters indicate that the means are significantly different at $P<0.05$.

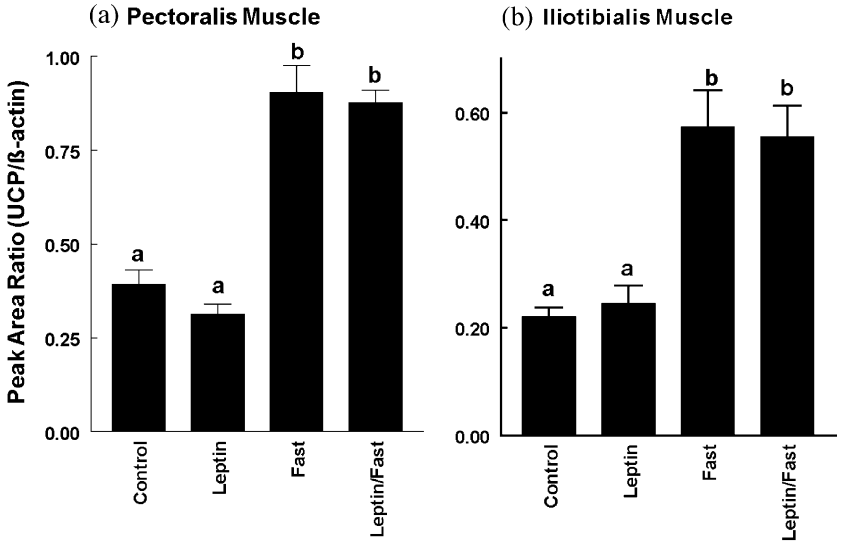

Fig. 4. Effect of feed deprivation for 48 h with or without exogenous rh leptin administration on changes in chicken UCP gene expression as measured by the integrated peak area ratio of UCP/β-actin in either (a) pectoralis major or (b) iliotibialis muscle. Values are plotted as mean ± standard error ($n=5$). Error bars with different letters indicate that the means are significantly different at $P<0.05$.

triglyceride and $r=0.79$ for NEFA). Plasma insulin concentration correlated with plasma IGF-I ($r=0.78$), IGF-II ($r=-0.45$) and pectoralis muscle IGF-I gene expression ($r=0.68$), but not with IGF-I receptor. Plasma insulin levels were also correlated with glucagon ($r=-0.54$) and chicken leptin ($r=0.52$) concentrations. Plasma glucose, lactate and glucagon all shared significant correlation. The levels of plasma IGF-I and IGF-II were negatively correlated with each other ($r=-0.50$), and IGF-I was also correlated with plasma glucagon ($r=-0.52$) and chicken leptin ($r=0.45$) concentrations. Plasma IGF-I was negatively correlated with pectoralis muscle IGF-I receptor gene expression ($r=-0.68$; Table 2) and positively correlated with muscle IGF-I mRNA level ($r=0.73$).

4. Discussion

Recently, expression of an avian UCP was documented in skeletal muscle of chickens and ducks (Raimbault et al., 2001), and was also found in skeletal muscle, heart, liver, lung and kidney of hummingbirds (Vianna et al., 2001). Data included herein using RT-PCR confirm the presence of an

Table 1
Effect of leptin and/or feed-deprivation on plasma hormone and metabolite concentrations

Metabolite	Control	Leptin	Starved	Leptin + starved	PSEM	ANOVA (P value)		
						F	L	F×L
Triglyceride (mg/dl)	34.2[a]	52.6[b]	17.4[c]	16.8[c]	2.3	0.0001	0.001	0.0007
NEFA (μeq/l)	139.1[a]	143.5[a]	395.9[b]	405.9[b]	28.0	0.0001		
Glucose (mg/dl)	240.8	247.1	241.1	235.6	7.1			
Lactate (mg/l)	260.0	419.6	295.2	370.0	53.0			
Insulin (ng/ml)	1.58[a]	2.69[b]	0.91[a]	1.29[a]	0.26	0.001	0.01	
Glucagon (pg/ml)	387.2	176.2	273.8	254.3	60.4			
Chicken leptin (ng/ml)	7.14[ab]	8.36[a]	6.14[b]	7.86[a]	0.48		0.008	
Human leptin (ng/ml)	0.0[a]	33.5[b]	0.0[a]	46.1[c]	1.4	0.0003	0.0001	0.0003
IGF-I (ng/ml)	45.2[a]	51.7[b]	22.4[c]	27.1[c]	1.8	0.0001	0.008	
IGF-II (ng/ml)	69.5[a]	79.6[a]	98.8[b]	97.4[b]	5.9	0.001		

F, feed deprivation main effect; L, leptin main effect; F×L, feed deprivation + leptin interaction.
[a bc]Values with different superscripts across a row are significantly different at $P<0.05$.

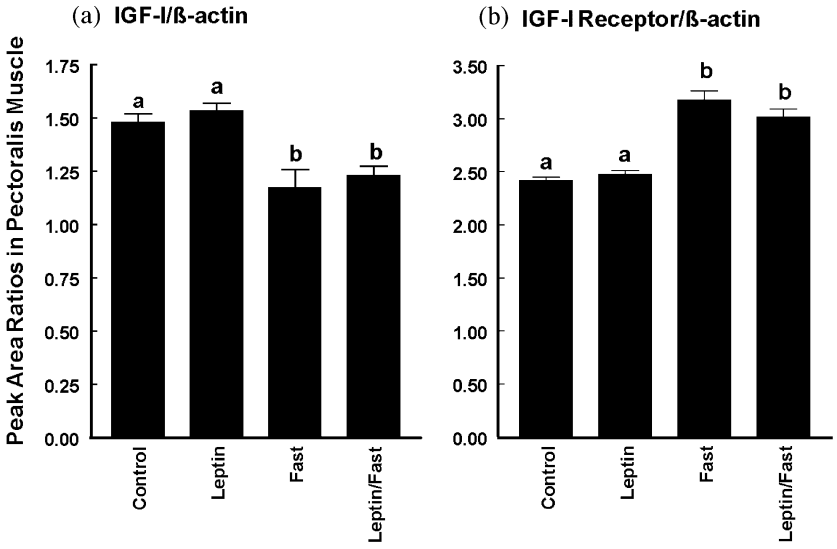

Fig. 5. Effect of feed deprivation for 48 h with or without exogenous rh leptin administration on changes in chicken (a) IGF-I or (b) IGF-I receptor gene expression as measured by the integrated peak area ratio of IGF-I or IGF-I receptor/β-actin in pectoralis major muscle. Values are plotted as mean ± standard error ($n=5$). Error bars with different letters indicate that the means are significantly different at $P<0.05$.

avian UCP gene homolog in skeletal muscle, heart, liver, kidney, adipose tissue, spleen, brain and lung tissue in chickens. Raimbault et al. (2001) may not have observed avian UCP in other tissues due to differences in thermocycling techniques compared to this study. In addition, it has been shown in rats that UCP 3 gene expression declined with age by up to 50% in glycolytic muscle and 15% (though not significant) in oxidative muscle (Barazzoni and Nair, 2001). The chickens used by Raimbault et al. (2001) were 62 weeks old, compared with the 8-week-old birds used in the present experiment. Clearly, more studies are required to accurately define the pattern of tissue expression of the UCP gene in birds.

In the chicken, the distribution pattern of UCP expression is similar to mammalian UCP 3, in that it is highly expressed in skeletal muscle, and has also been reported in human heart and liver (Acin et al., 1999). Interestingly, Acin et al. (1999) observed that human liver UCP 3 expression was quite low and was present only when primers from the coding region were used to examine its expression; two primers located in the 5′ untranslated

Table 2
Normal correlations (with P values in parentheses) between tissue UCP, IGF-I and IGF-I receptor gene expression and all other parameters measured in Experiment 3

Item	Pectoralis UCP	Iliotibialis UCP	IGF-I	IGF-I receptor
Iliotibialis UCP	0.847 (0.0001)		−0.680 (0.001)	0.666 (0.002)
Pectoralis IGF-I	−0.801 (0.0001)	−0.680 (0.001)		−0.672 (0.002)
Receptor	0.640 (0.003)	0.666 (0.002)	−0.672 (0.002)	
Triglyceride	−0.866 (0.0001)	−0.792 (0.0001)	0.831 (0.0001)	−0.682 (0.001)
NEFA	0.701 (0.001)	0.774 (0.0001)	−0.680 (0.001)	0.792 (0.0001)
Glucose	−0.238	−0.119	0.084	−0.060
Lactate	−0.137	0.117	−0.078	0.178
Glucagon	0.556 (0.01)	0.239	−0.445	0.195
Insulin	−0.847 (0.0001)	−0.710 (0.0005)	0.681 (0.001)	−0.365
Chicken leptin	−0.460 (0.04)	−0.385	0.222	−0.236
Human leptin	0.016	0.207	−0.074	0.146
IGF-I	−0.901 (0.0001)	−0.802 (0.0001)	0.729 (0.0003)	−0.679 (0.001)
IGF-II	0.609 (0.005)	0.580 (0.01)	−0.647 (0.002)	0.618 (0.004)

Table 3
Normal correlations (with *P* values underneath) between all plasma parameters measured in Experiment 3

Item	Triglyceride	NEFA	Glucose	Lactate	Glucagon	Insulin	Leptin		IGF-I	IGF-II
							Chicken	Human		
Triglyceride		−0.755 0.0001	0.220	0.039	−0.487 0.03	0.730 0.0003	0.410	−0.078	0.791 0.0001	−0.644 0.003
NEFA	−0.755 0.0001		−0.206	0.056	0.302	−0.451 0.05	−0.311	0.273	−0.741 0.001	0.564 0.01
Glucose	0.220	−0.206		0.553 0.02	−0.710 0.001	0.162	0.592 0.006	0.082	0.331	0.048
Lactate	0.039	0.056	0.553 0.02		−0.663 0.002	0.204	0.517 0.02	0.469 0.04	0.201	0.113
Glucagon	−0.487 0.03	0.302	−0.710 0.001	−0.663 0.002		−0.542 0.02	−0.704 0.0005	−0.380	−0.517 0.02	0.090
Insulin	0.730 0.0003	−0.451 0.05	0.162	0.204	−0.542 0.02		0.525 0.02	0.315	0.776 0.0001	−0.454 0.05
Leptin	0.410	−0.311	0.592	0.517	−0.704	0.525		0.565	0.453	−0.041
Chicken			0.006	0.02	0.0005	0.02		0.01	0.05	
Human	−0.078	0.273	0.082	0.469	−0.380	0.315	0.565		0.034	0.284
IGF-I	0.791 0.0001	−0.741 0.001	0.331	0.201	−0.517 0.02	0.776 0.0001	0.453 0.05	0.034		−0.499 0.03
IGF-II	−0.644 0.003	0.564 0.01	0.048	0.113	0.090	−0.454 0.05	−0.041	0.284	−0.499 0.03	

region did not detect human liver UCP 3. Similarly, our pattern of expression of both the major amplicon of chicken UCP and other size fragments, appearing in tissues other than skeletal muscle (Fig. 1), can be altered using different primer sets from different locations within the gene (data not shown). Although we could not sequence the > 1000-bp band, it is interesting to speculate that some of the other bands present might represent other chicken UCP homologs or splice variants, especially since UCP 2 and UCP 3 share high sequence homology among mammals. In humans, both a long and short form of UCP 3 have been identified (Solanes et al., 1997). In addition, the two most recently discovered UCPs, UCP 4 and BMCP 1, are mainly brain-specific, and brain does exhibit more amplicons than any other tissue. The chicken UCP tissue-expression pattern is also similar to mammalian UCP 2, in that it is ubiquitous, although at much lower levels of expression than in skeletal muscle. Both the nucleotide (Table 4) and amino acid sequences of chicken UCP show slightly higher homology to mammalian UCP 3 than to UCP 2. Experimentally, it would be difficult to unambiguously determine if chicken UCP is a homolog of UCP 3 or UCP 2, due to the similarities in their responses to a number of stimuli (Table 5). However, there are differences

Table 4
Overlapping nucleotide sequence identities between chicken and other avian UCPs, and mammalian UCP 2 and UCP 3

Avian species	UCP (%)	GenBank #	UCP 2	GenBank #
Meleagris gallopavo (turkey)	94	AF436811		
Eupetomena macroura (hummingbird)	84	AF255729		
Other vertebrate species	UCP 3			
Sus scrofa (pig)	74	AF095744	74	AF036757
Canis familiaris (dog)	73	AB022020	71	AB020887
Homo sapiens (human)	73	AF050113	70	AF096289
Bos taurus (cow)	74	AF092048	72	AF127029
Rattus norvegicus (rat)	72	AF035943	71	AF039033
Mus musculus (mouse)	73	AF053352	74	AF111999
Phodus sungorus (hamster)	72	AF271265	71	AF271264

Table 5
Effects of different parameters on UCP 2 and UCP 3 gene expression in skeletal muscle

Parameter	UCP 2	UCP3	Reference
Cold exposure, acute, chickens		U	Raimbault et al., 2001
High fat diet, mice	U	+	Gong et al., 1999
Diabetes, rats	++	+	Kageyama et al., 1998
Food restriction (50%), rodents		−	Boss et al., 1998
Feed deprivation, humans, pigs	+	+	Millet et al., 1997; Spurlock et al., 2001
Refeeding, rodents, early		−	Marzolo et al., 2000
Transgenic knockout mice, LPL	U	−	Kratky et al., 2001
Fatty acids, rats	+/U	++	Weigle et al., 1998
T3, human, rats	+	+	Barbe et al., 2000; Short et al., 2001
Leptin, mice, acute	U	−	Combatsiaris and Charron, 1999

+, increased; ++, highly increased; U, unchanged; and −, decreased.

in the promoter regions of mammalian UCP 3 and UCP 2. Both mouse (Yoshitomi et al., 1998) and human (Acin et al., 1999) UCP 3 contain a putative TATA box, while mouse (Yamada et al., 1998) and human (Pecqueur et al., 1999) UCP 2 do not. Sequencing the 5′ region of the avian UCP gene might help to determine its true identity.

Feed deprivation in chickens was associated with a time-dependent increase in skeletal muscle UCP gene expression, regardless of fiber type. This association was also reported in pigs (Spurlock et al., 2001) for UCP 3 only, and rats (Samec et al., 1998) for both UCP 2 and UCP 3. Feed deprivation in chickens also induced metabolic changes, such as increased NEFA levels with a concomitant decrease in plasma triglyceride concentration, in response to an increase in overall fatty acid oxidation. These plasma components were both strongly correlated with chicken muscle UCP mRNA levels. These high correlations lend support to the recent hypotheses that free fatty acids have a role in up-regulating both UCP 2 and UCP 3 expression in skeletal muscle, with UCP 3 regulating lipid flux by serving as a carrier protein to transport free fatty acids out of the mitochondria (Himms-Hagen and Harper, 2001), and UCP 2 reducing lipotoxicity by controlling excess production of reactive oxygen species in response to the increased flux of lipid substrates (Dulloo and Samec, 2001). In addition to feed deprivation, which elevates NEFA levels, feeding a high-fat diet to human males (Schrauwen et al., 2001) induced UCP 3 expression in muscle, and infusing fed rats with a lipid emulsion also led to an increase in UCP 3 expression (Weigle et al., 1998). This effect can also be observed in vitro, where addition of oleic acid to differentiated C2C12 myoblasts increased UCP 3 mRNA levels in a time- and concentration-dependent manner (Hwang and Lane, 1999). Addition of a lipid emulsion, oleic or linoleic acid to cultured rat hepatocytes resulted in increased UCP 2 gene expression and protein content (Cortez-Pinto et al., 1999), even after addition of glutathione, an antioxidant that is impermeable to cells. More recently, Huppertz et al. (2001) reported that human UCP 3 expressed in L6 myotubes or in H(9)C(2) cardiomyoblasts increased 2-deoxyglucose uptake two-fold, while overexpression of UCP 3 in 3T3-L1 adipocytes did not affect glucose uptake. However, this increase in glucose oxidation was inhibited by addition of oleate to cultured human muscle cells (Garcia-Martinez et al., 2001), while addition of glucose could not suppress the increased oleate oxidation caused by overexpression of UCP 3. In addition, Son et al. (2001) have reported that at least part of the increase in fatty acid-mediated UCP 3 gene expression in muscle cells in culture is activated through PPAR-δ, a known fatty acid agonist. These data indicate that UCP 3 has tissue-specific effects, suggesting a possible role in nutrient partitioning under times of metabolic stress.

Feed deprivation in chickens also induced a decrease in plasma IGF-I and insulin, with a concomitant increase in IGF-II concentrations. In addition, IGF-I gene expression in skeletal muscle paralleled the decrease in plasma IGF-I and insulin concentrations, while muscle IGF-I receptor gene expression was proportionately increased. All the IGF components and insulin were also strongly correlated with chicken muscle UCP mRNA levels. This is the first study showing a possible link between the IGFs, insulin and skeletal muscle

UCP in birds. Correlations themselves do not imply a causal relationship. However, the IGFs do appear to have a role in regulating lipid metabolism in poultry. Administration of exogenous rh IGF-I to chickens resulted in a decrease in abdominal fat mass (Huybrechts et al., 1992), while treatment with rh IGF-II had the opposite effect (Spencer et al., 1996). In vivo administration of either IGF-I or IGF-II to chickens has been shown to increase free fatty acid concentrations without affecting triglyceride levels (McMurtry et al., 1996). Additional support for a relationship between IGF-I and UCP was reported by Gustafsson et al. (2001). They showed that IGF-I added to human neuroblastoma cells in culture up-regulated the expression of UCP 3 via the IGF-I receptor. And, interestingly, Duchamp et al. (1997) reported that chronic cold exposure up-regulated IGF-I gene expression in brown adipose tissue in rats, a condition that also increased the levels of UCP 2 and UCP 3 mRNAs in brown adipose tissue (Denjean et al., 1999).

Unlike most mammals, there were no observable differences in plasma glucose, lactate or glucagon levels from feed-deprived birds vs. those on ad libitum feeding in this study. Glucose concentration in chickens is tightly regulated by insulin and glucagon, and possibly by IGF-I as well (McMurtry et al., 1997).

The avian UCP homolog was originally reported to have a role in adaptive thermogenesis, based on increased UCP expression in skeletal muscle during cold exposure or glucagon administration in ducks (Raimbault et al., 2001), or during torpor in hummingbirds (Vianna et al., 2001). This would seem to be in direct opposition to our observation of an increase in avian UCP during feed deprivation, a situation involving decreased energy expenditure. However, Dulloo and Samec (2001) suggested that most stimuli used to induce thermogenic responses, such as cold exposure and high-fat feeding, or the administration of T3, β-adrenergic agonists or leptin (Gong et al., 1997), also enhance the utilization of lipids as fuel substrates. Further evidence against a true uncoupling role for UCP 3 was the finding by Jucker et al. (2000) that skeletal-muscle mitochondrial energy coupling did not change in fasted vs. fed rats, while UCP 3 mRNA expression increased. In addition, Iossa et al. (2001) found no relationship between UCP 3 protein content and basal proton leak kinetics in skeletal muscle mitochondria from either fed or fasted rats.

Although rh leptin was present in chicken plasma, it had no effect on feed intake over the 2 days of this experiment. Mouse recombinant leptin (5 μg) also did not reduce feed intake in chickens 24 h after an i.c.v. injection (Bungo et al., 1999). Intraperitoneal injection of 1 mg/kg body weight of chicken leptin to chickens reduced feed intake between 2 and 8 h post-injection, but this effect disappeared 9–10 h after treatment (Dridi et al., 2000). Rh leptin treatment also had no effect on avian UCP expression in either the fed or fasted state in the present study. Gomez-Ambrosi et al. (1999) reported that leptin increased muscle UCP 3 expression 1 h post-treatment; however, their rats were fasted for 24 h prior to leptin administration. On the other hand, Combatsiaris and Charron (1999) observed a 35% decrease in muscle UCP 3 expression and a 50% decrease in muscle UCP 2 expression 5 h after leptin administration in mice. The dosage, frequency, time course and mode of delivery of exogenous leptin administration may all have effects on changes in muscle UCP expression that were undetectable in this study. This needs to be examined in more detail to determine if and/or when leptin influences UCP expression in muscle of normal animals.

Recombinant human leptin administration to chickens did increase plasma triglyceride concentrations, but had no effect on NEFA levels in ad libitum fed birds. Wang et al. (1999) also reported that leptin caused triglyceride depletion from rat adipocytes in culture, with an increase in glycerol release but no concurrent increase in FFA release. Exogenous leptin treatment increased chicken leptin levels in fasted birds, while plasma IGF-I and insulin concentrations were increased in rh leptin-treated fed birds. Gomez-Ambrosi et al. (1999) also observed no effects 1 h after a single injection of leptin on either plasma glucose or insulin concentrations in 24-h fasted rats. Leptin administered i.c.v. to fed pigs had no effect on serum insulin, glucose, FFAs or IGF-I (Barb et al., 1998). Rh leptin concentration in plasma did not correlate with gene expression of the chicken leptin receptor in skeletal muscle (data not shown).

In summary, we report clear evidence that the metabolic adaptation to feed deprivation in chickens leads to an increase in skeletal muscle UCP mRNA, and that this increase in UCP is highly correlated with plasma triglyceride, NEFA, insulin,

IGF-I and IGF-II concentrations, as well as with IGF-I and IGF-I receptor gene expression in skeletal muscle. Recombinant human leptin had no effect on UCP expression in skeletal muscle in birds, either in the fasted or the fed state. Although the exact biochemical role or mechanism of action of this UCP is currently unknown, it is likely that avian UCP is involved in regulating, or is regulated by fatty acid oxidation, especially at times of nutritional stress.

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